S E C R E T

16 February 1970

MEMORANDUM FOR: Chief, Clinical Division, OMS

SUBJECT : Information From USAF - School of Aviation Medicine

1. Lipo-Protein Determination

Use Beckman Microzone with Gelman's Sepraphore III Paper and cellulose acetate.

Uses of Selman's Procedure using cyclo-hexanone mixed with ethanol.

Procedure is in Gelman's Book on Clinical Pathology. Gelman is at P.O. Box 1448, Ann Arbor, Michigan. Latest article on this technique is in A.M. Society Clinical Pathologists (ASCP) Journal, January 7, 1970, (Electrophoresis).

2. Triglyceride Determination

Apparently there have been many changes in techniques in past few years.

Mrs. Dorothy Wease, who has been at the SAM Laboratory for many years, is somewhat of an authority on this procedure.

Her procedure is her own modification using the Technicon Analyzer (Technicon Procedure # 78). (This has been found very satisfactory over past few years.)

The enclosed Blue Book has the 1966 method for extraction. After this first step, it is all done in auto-analyzer. (The old method utilized a zealite mixture in extracting which is not satisfactory.)

S E C R E T

At present time manifold tube on analyzer will not handle ether; therefore, change procedure to use isopropanol. Isopropanol does not get all lipoproteins back in solution; therefore, a small amount of ether is added at the end to accomplish this.

The phospho-lipids are lost in this procedure. Cholesterol and triglycerides are run on same sample. The method for this procedure is attached.

The method for determining phospho-lipids is now in print and Mrs. Wease will send it to me as soon as completed.

3. If necessary, it can be easily arranged to have a tech go to San Antonio and spend whatever time is necessary at their laboratories.

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FS/OMS

Attachments
1 - Copy of Lipoprotein Electrophoresis Procedure
2 - Copy of Triglyceride Procedure

LIPOPROTEIN AND GLYCOPROTEIN ANALYSIS

LIPOPROTEIN ELECTROPHORESIS

Many small molecules are transported in blood in combination with plasma proteins. Lipoprotein is an example of such a complex. The lipoproteins contain such lipids as free cholesterol, cholesterol esters, phospholipids, and mono-, di- and tri-glycerides.

Electrophoresis is a valuable aid in diagnosing several specific lipoprotein deficiency states and in characterizing lipoproteinemias. It is much more convenient and economical than ultracentrifugation and is adaptable to the screening of many subjects at relatively low cost. Visual inspection of the electrophoregram is presently employed to check for normals and some abnormals of specific types. The Gelman densitometric procedures now allow quantitation of lipoprotein electrophoregrams in only a slightly longer time.

The normal fasting plasma lipoprotein electrophoregram should contain three bands. These bands are located in the α_2 pre-β and β-globulin region. A fourth band will be located at the origin if chylomicrons are present.

Two staining procedures are presented. One involves lipid staining with Oil Red O. The alternate method involves lipid oxidation followed by staining with a modified Schiff's reagent as proposed by Kohn (1961). This method gives stained bands that coincide with those obtained by the Oil Red O method.

A. Equipment and Supplies

Same as for the basic serum protein procedure

Oil Red O Lipid Stain Procedure:

Oil Red O, C.I. (Fisher Chemical No. NA-689; Harleco No. 3125

Commercial Liquid Bleach (approximately 5.25% sodium hypochlorite)

Ozone-Schiff Stain Procedure:

Schiff's Reagent—Fuchsin Sulfurous Acid for the detection of aldehydes (Harleco No. 2818)
Nitric Acid
Sulfuric Acid

Barium Dioxide (BaO_2)
EDTA (tetra-sodium salt of ethylenediaminetetra-acetic acid)
Beaker, 2000 ml
Aluminum Foil

B. Specimen

Use a fasting-state blood sample. Collect 5 ml blood and gently mix in a tube containing 1.0 ml of 0.001 M EDTA in 0.15 M NaCl. Use low speed centrifugation to separate the plasma. Remove plasma and store at 1° C to 3° C. Under these conditions lipoproteins are stable for at least 2 weeks as judged by electrophoregram reproducibility (Kelleher, 1968). EDTA is recommended to prevent the formation of oxidation products from the normally present lipoproteins. This oxidation occurs very rapidly in the presence of catalytic concentrations of divalent cations, giving an electrophoregram showing six bands rather than the normal four bands.

C. Reagent

1. Buffer—300 ml (100 ml for tray and 200 ml for SepraTek Chamber). Dissolve 1 packet of High-Resolution Buffer in distilled water and dilute to 670 ml. Some clinicians add 0.685 gm EDTA to the buffer (0.001 M concentration) to prevent problems with divalent cations.

2. Stain—1.0 gm Oil Red O in 1000 ml warm 70% methyl alcohol. Bring to a boil for 5 minutes. Then, store at 37° C until used.

D. Electrophoresis Procedure

1. Carry out electrophoresis as outlined for the serum protein procedure (Steps 1-15) except make 3 or 4 applications by repeating Step 12.

2. Electrophorese at 200 volts for 30 minutes.

3. When the run is over, turn off and disconnect the Power Supply. Remove Sepraphore strip holding it horizontally taut. Do not allow buffer to run over the strip.

E. Oil Red O Staining Procedure

Follow equipment, reagents, and specimen preparation given above.

1. Place damp electrophoregram into 100 ml of the Oil Red O stain for 14-24 hours at 37° C. If more than one strip is stained at one time place the strips into the staining tray in such a way as to prevent one strip from overlapping any other. Maintain the 37° C temperature by placing the staining tray in an incubator.

2. After 24 hours, rinse the strip(s) in 100 ml of distilled water.

3. Decolorize background in a hypochlorite solution composed as follows: 1 ml commercial laundry bleach (5.25% sodium hypochlorite) to 200 ml aqueous, 5% acetic acid. Five to ten minutes is sufficient to lighten the background.

4. Store in aqueous 5% acetic acid until ready for clearing and quantitation.

F. Alternate Procedure (Modified Schiff's Reagent)

1. Ozonation—DO IN A WELL VENTILATED HOOD. Wrap a piece of insulated wire around the periphery of a 2000 ml beaker and near the top. String wires across the top of the beaker to form "clothes lines" (a "W" shape) using the periphery wire as anchor points.

Place 10-15 gm BaO_2 into a 250 cc side arm flask (Pyrex). Connect a piece of tubing to the side arm. Have a base of sufficient length that the free end can be placed into the beaker.

Suspend the electrophoresed strip from the ozonator wire rack and clamp with a paper clip. Cover the top of the beaker with a piece of aluminum foil. Add 20 to 25 ml concentrated H_2SO_4 to the side arm flask and stopper immediately. Ozone is carried over to the beaker via the tubing. Continue for 10 minutes. Additional H_2SO_4 may be added should gas production decrease before 10 minutes has passed.

2. Remove the strip(s) from ozonator. Place into 0.001 N HCl rinse solution for one minute. If more than one strip is stained at one time, do not allow strip(s) to overlap, because some of the lipoprotein may diffuse onto or contaminate another strip, causing irregular band shapes and patterns.

3. Staining and Decolorizing. Place the strip(s) into the Schiff's reagent trays. (Do not allow extra strips to overlap.)

   Keep the stain-containing trays covered with aluminum foil to exclude light. The stain reagent may be used a number of times. It should be discarded when it begins turning pink.

   Stain for 30 to 60 minutes to develop maximum blue-purple color.

4. Wash. Carefully remove the stained electrophoregram and wash in 3 successive baths of 0.5% aqueous nitric acid.

5. Store in the final rinse until ready for clearing and quantitation.

G. Clearing and Quantitating

1. After employing either the Schiff's Reagent or the Oil Red O staining technique, remove the

electrophoregram from the final rinsing solution. Blot the strip(s) between two dry blotter pads until only the excess moisture is removed. The strip(s) should still feel damp to the touch. It is imperative, particularly with the Schiff's Reagent technique, that the strip not be completely dry since not only will the bands fade, but also, the clearing solution will destroy the membrane.

2. Place the damp membrane into a clearing solution composed of either 30% dimethyl sulfoxide (DMSO) in water or 30% aqueous dimethyl formamide (DMF) for 5 minutes.

3. Remove the strip from the clearing solution and place on a 2.25" x 2.75" glass slide. Place the strip and the glass slide into an oven set at 100° C for 5 to 10 minutes. Remove the strips as they clear. Prolonged overheating yields brittle, curled strips that are difficult to handle.

4. Scan at 545-555 nm for the Schiff's technique and 525 nm for the Oil Red O technique.

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Triglyceride Procedure

1. *Reagents: Silicic Acid (BIO-SIL HA minus 325 mesh)

2% KOH

Acetyl Acetone

Periodate with 3% Acetic Acid

Isopropyl Alcohol (Redistilled)

2. Procedure:

(1) Into 50 ml round bottom pyrex tubes (with glass stoppers), pipette 1 ml standards, controls, serum.

(2) Add 2.5 g silicic acid to each tube and stopper.

(3) Let stand 15 minutes.

(4) Hit tubes sharply on padded surface to shake silicic acid and serum loose from the sides of the tubes (Do not break tubes.).

(5) Add 25 ml ether; stopper, shake once by hand, and remove pressure.

(6) Shake in horizontal action shaker 10 minutes.

(7) Centrifuge standards for 1 minute at 5000 rpm. (Standards take longer to settle out than do other samples because of less water).

(8) Pipette a 5 ml aliquot from each tube into another 50 ml tube.

(9) Remove stopper and dry in 60°C water bath. (About 15 min.)

(10) Remove and cool at room temperature. not necessary to cool

(11) Add 0.5 ml ether; stopper and mix to dissolve lipid.

(12) Add 5.0 ml Isopropyl alcohol; stopper, and mix thoroughly.

(13) Let stand 15-20 minutes before placing on sampler.

(14) Pour aliquot into AutoAnalyzer cup, and place cup into covered sample wheel. Evaporation may lead to significant errors if the cups are left in the sample wheel for longer than an hour.

3. *Reagent Preparation:

(1) 2% KOH

20 grams KOH

Dissolve in distilled water and qs to 1000 ml

(Stable for 1 week)

(2) Periodate with 3% Acetic Acid

0.53475 grams sodium periodate

15 ml 3% Acetic Acid

Mix Acetic Acid with about 300 ml distilled water, add Periodate and mix well. QS to 500 with distilled water.

(Stable for 1 day)

(3) Acetyl Acetone

25 ml Isopropyl Alcohol

2M Ammonium Acetate

Put 25 ml Isopropyl Alcohol in a 1 L flask and qs with Ammonium Acetate and mix well. Add 7.5 ml Acetyl Acetone and mix thoroughly. Let stand until yellow color develops (5-10 minutes).

(Stable for 1 day)

Standards are purified Tripalmitin of appropriate concentration dissolved in Isopropyl Alcohol. The standard solutions are carried through the complete extraction procedure.